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ORIGINAL ARTICLE

Neuroprotective effect of ethanol and Modafinil on focal cerebral ischemia in rats

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Abstract

Ethanol is known as an effective agent against cerebral lesions after ischemia. Modafinil is a stimulant of the central nervous system (CNS) with antioxidant properties. We assessed the neuroprotective effect of modafinil in combination with ethanol after focal cerebral ischemia. Male wistar rats weighing 280–300 g were divided into nine groups ($n = 12$ each group): The groups consisted of the MCAO (middle cerebral artery occlusion) group (i.e. ischemia without treatment); the vehicle group(Dimethylsulfoxide); the modafinil group including three subgroups which pretreated with Modafinil (10, 30, 100 mg/kg), respectively, for seven days prior to the induction of MCAO; the ethanol group which received 1.5g/kg ethanol at the time of reperfusion; and modafinil+ethanol group which was divided into three subgroups that received three doses of modanifil (10, 30,100 mg/kg), respectively, for seven days prior to MCAO as well as ethanol at the time of reperfusion. Transient cerebral ischemia was induced by 60-min intraluminal occlusion of the right middle cerebral artery. Edema, infarct volume, glial scar formation (gliosis) and apoptosis were analyzed. The ethanol alone treatment (with a less significant effect), modafinil (in a dose-dependent way), and the combination of modafinil and ethanol significantly decreased the brain infarct volume, edema, apoptosis, and gliosis ($P \leq 0.05$). Additionally, modafinil+ethanol mediated the restoration of aerobic metabolism and hyper-glycolysis suppress, thereby resulting in an increase in pyruvate dehydrogenase and a decrease in lactate dehydrogenase activity, respectively, which ultimately reduced oxidative reperfusion injury. These results demonstrate that pretreatment with modafinil (100 mg/kg) and modafinil+ethanol(1.5 g/kg) may prevent ischemic brain injuries.

Keywords Neuroprotection · Ethanol · Modafinil · Cerebral ischemia · Rat

Introduction

Stroke is the fifth and third leading cause of mortality in the US and the world, respectively (Chen et al. 2016; McCarter et al. 2017a). A series of events are involved after middle cerebral artery occlusion specified by biochemical and histopathological consequences which eventually lead to cell swelling and neuronal death (Ghahari et al. 2014). The Food and Drug Administration (FDA) approved a number of thrombolytic medications such as those classified as tissue plasminogen activator (tPA). Notably, an intra-arterial therapy (IAP) is currently used for the treatment of acute ischemic stroke (Kochanski et al. 2013; McCarter et al. 2017a). These approaches, because of the limited therapeutic efficacy, are useful only for a small percentage of patients (Kochanski et al. 2013). Numerous studies over the past several decades have demonstrated that significant therapeutic advances have made it possible to design neuroprotective compounds (Beraki et al. 2013) However, there is not any approved medicine to meet the required criteria to be used in clinical trials and prescribed for patients with cerebral ischemia.

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Ethanol is a potential neuroprotective agent for the treatment of acute ischemic stroke, causing no serious effect on intracerebral hemorrhage when used with thrombolytics (Wang et al. 2012). It is readily available, well-tolerated, and easy to be administered since it diffuses across the blood-brain barrier (BBB) (Cai et al. 2016a). Moderate ethanol consumption has protective effects on apoptotic cell death by decreasing the level of caspase-3 enzyme activity, inhibition of lysosomal protease release, and production of nitric oxide after traumatic brain injury (Kanbak et al. 2013). Combination of ethanol and normobaric oxygen (NBO) decreases Glucose transporter1 (Glut1), Glucose transporter3 (Glut3), Phosphofructokinase-1(PFK-1), and lactate dehydrogenase (LDH) levels and increases pyruvate dehydrogenase (PDH) which can potentially reduce post-stroke hyper-glycolysis(Cai et al. 2016a). Ethanol administration, prior within 3 to 24 h after the reperfusion significantly decreases the expression of pro-apoptotic protein PKC-δ while increasing the anti-apoptotic protein Akt at the levels of both mRNA and its cognate protein (Hafeez et al. 2014).

Modafinil is a stimulant of the central nervous system (CNS) approved by the US Food and Drug Administration (FDA) and possesses a neuroprotective effect through increasing antioxidant properties(Dias et al. 2017). Modafinil helps to prevent inflammation and loss of dopaminergic neurons in substantia nigra in animal models of Parkinson's disease (PD) (Brandt et al. 2014; Raineri et al. 2011; van Vliet et al. 2006; Zager et al. 2018). The combined effect of modafinil and caffeine on sleep disorders in the hippocampal dentate gyrus showed that modafinil and caffeine are cooperatively able to increase the expression of the brain-derived neurotrophic factor (BDNF) as well as the level of neurogenesis doublecortin (DCX) cells (Sahu et al. 2013). Modafinil prevents the increase of a pro-apoptotic factor called BAX and the decrease of an antiapoptotic factor named B cell lymphoma 2 (Bcl-2). It also reduces the astrocyte and microglia activated (gliosis) by methamphetamine in the striatum of rats (Raineri et al. 2012). Moreover, it is capable of enhancing the quality of life in stroke survivors (Bivard et al. 2017). Modafinil improves the cognitive and motor ability in patients with multiple sclerosis (Lange et al. 2009). Modafinil consumed at doses of 10, 30, and 100 mg/kg diminishes the lesion volume, lactate level (tissue acidosis), and behavioral deficits in animal models with endothelin-1-induced ischemia (Ueki et al. 1993). Since co-administration of antioxidants may have synergistic effects, so we designed this study.

Materials and methods

Animals

In this study, a total of 108 adult male wistar rats weighing 280–300 g were used. The animals were kept under the standard condition (22 °C–24 °C, 45–50% humidity, 12 h light–dark cycle) with free access to food and water. The rats were randomly divided into nine groups ($n = 12$ in each group) as follows:

Experimental groups included the middle cerebral artery occlusion group (designated as MCAO); the vehicle group designated as Veh;, the modafinil group which was in turn divided into three subgroups that received modanifil at the doses of 10,30 and100mg/kg (designated as M10, M20, and M100), respectively, for 7 days prior to MCAO(Ueki et al. 1993); the ethanol group (designated as E) which received 1.5g/kg ethanol at the time of reperfusion(Hafeez et al. 2014); and the Modafinil+ethanol group which was divided into three subgroups that received modafinil at three doses of 10, 30, and 100 mg/kg (designated as E + M10, E + M30, and E + M100) for 7 days prior to MCAO as well as ethanol at the time of reperfusion.

MCAO model

The rats were anesthetized by means of intraperitoneal injection of 10% chloral hydrate)Merck, Germany) (400 mg/kg) (Maleki et al. 2018). Right common carotid as well as the external and internal carotid arteries were exposed through a midline incision in the neck under a surgical microscope (Olympus Szx12, Germany). MCA occlusion was performed by inserting a silicone coat filament (Doccol Corp., Sharon, MA, USA) in the right internal carotid artery via the external carotid artery until it reached the anterior cerebral artery (Mokudai et al. 2000; Sicard and Fisher 2009). The filament was located in the internal carotid artery for 60 min and then removed. Ethanol (1.5 g/kg) was injected immediately after the removal of the obstruction, and the reperfusion was performed in both the E group and the E + M group. Modafinil (Dipharma- Italy) was dissolved in DMSO(Bezu et al. 2016) and then injected for seven days at 8:00 A.M except for the last injection which was carried out half an hour before the induction of the ischemic model. The body temperature of animals was monitored with a rectal thermometer (Kent Scientific Corporation, USA) and maintained at 37 °C while a 220 V lamp was placed next to the animals.

Quantification of infarct volume

The animals were killed under deep anesthesia within 24 h after MCAO. The brains were quickly removed and cooled in iced saline for 10 min and then cut into 2-mm thick coronal sections using the brain matrix (Stoelting Co, USA). The sections were incubated with 2% of 2,3,5-triphenyl tetrazolium chloride(Sigma, Germany) at room temperature for 20 min and then fixed in 10% buffered formalin)Merck, Germany) solution. A digital camera (Canon, Japan) was used to take photographs of the slices. The Image J software v1.8 (NIH, Wayne Rasband, USA) was applied for the measurement and analysis of the infarct volume. To minimize error causing the edema, the infarct volume was calculated by an indirect method (Wang et al. 2012). The area of each slice was multiplied by its thickness (2 mm). Subsequently, the infarct volumes (mm^3) of individual slices were summed up to achieve the total infarct volume of the hemisphere for each ischemic rat brain. Finally, the samples were expressed as a percentage in comparison with the non-infarcted contralateral cerebral hemisphere.

Brain water content determination

A wet/dry method in different groups was used for determination of the brain water content. For this aim, rats were over-dosed using injection of chloral hydrate within 24 h after reperfusion and then decapitated. The brains were carefully removed and precooled and the brain matrix was employed for the removal of the olfactory bulb, cerebellum, and brain stem. The brains were split into ischemic and contralateral hemispheres. The ischemic hemisphere was weighed (wet weight) and placed in an oven at 110 °C for 24 h and again weighed (dry weight). The brain water content was determined via the following formula (wet weight - dry weight)/wet weight × 100%(Maleki et al. 2018).

Cresyl violet staining

After perfusion, the fixed samples were made of paraffin mold. After paraffin (Merck, Germany) embedding, coronal sections (7 μm) were prepared by using a rotatory microtome (Licka, USA). The sections were placed on the slides. After clearing and rehydration, the slides were stained with 0.1% crystalline violet. Finally, the specimens were covered with Entelan adhesive and slide cover. The stained slices were evaluated by 400x magnification (Labomed, USA). After taking the photos, the cells in peri-infarct areas were counted by Image J software v1.8 (NIH, Wayne Rasband, and USA). Cell shrinkage, loss of uniformity of Nissl body, cytoplasm, nucleus density, and pyknotic nucleuswere evaluated by a light microscope (Labomed, USA)(Zhang et al. 2015).

Terminal dexynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The in situ cell death detection was used to assess the DNA fragmentation using POD Kit (Roche, Germany). The histological sections were incubated with proteinase K (15 μg/ml) for half an hour. The sections were then incubated in 3% hydrogen peroxide/methanol for 10 min in the dark at room temperature to block endogenous peroxidase activity. After three times washing in Tris buffer (each 5 min), the sections were incubated with a TUNEL reaction mixture for 1 h at 37 °C. The sections were washed in Tris wash buffer 3 times for 5 min each and, then, incubated with POD for 15 min at 37 °C. Again, the sections were washed in Tris wash buffer 3 times for 5 min each and, then, color development was performed in the dark room with DAB (3,3-Diaminobenzidine) for 15 min. Afterwards, hematoxylin solution was used as counter stain. After washing in Tris wash buffer 3 times for 5 min, the number of TUNEL positive neurons was counted carefully in 3 sections of the peri-infarct area of the cortex per animal. Cells were defined as apoptotic cells if the entire nuclear area of the cell was positively labeled. The apoptotic cells and bodies were counted in five high-power fields(Enayati et al. 2018). The apoptosis index calculated as percentages percentage of positively stained cells using the following equation: AI = number of apoptotic cells/total number of nucleated cells(Liu 2018).

Immunohistochemistry

Immunofluorescent (IF) IHC staining was performed to determine Procaspase 3 proteins and IHC DAB staining was used to the GFAP (glial fibrillary acidic protein) protein expression. The rats were transcardially perfused with 4% paraformaldehyde in phosphate buffer and then killed. The brains of the rats were removed and postfixed overnight. Then, they were dehydrated in the ascending alcohol series, rinsed by xylene, and infiltrated with paraffin. Afterwards, all of the blocks were divided into 5 μm coronal sections. The sections were incubated in 50% formamide and 2x standard sodium citrate buffer at 65 °C for 2 h and then incubated twice in 100 mM of sodium borate (pH = 8.5). The DNA was

then denatured by incubating the sections in 2 N HCl at 37 °C, rinsed in phosphate-buffered saline (PBS), and blocked with 0.4% Triton X-100 in PBS and goat serum (10%) for 30 min. The sections were incubated overnight at 4 °C with primary antibodies for GFAP (rabbit anti-GFAP (1:100, Abcam, Cambridge, United Kingdom)) and procaspase3 (rabbit anti-procaspase3 (1:100, Abcam, Cambridge, United Kingdom)). The slices were then incubated with secondary antibody at 37 °C for 90 min in a dark place. Peroxidase-conjugated secondary antibodies were used for chromogenic detection by oxidizing 3,3′-Diaminobenzidin (1:200, ab205718) that was used for the primary antibody detecting as well as FITC anti-rabbit 488 (1:200; ab6717) that was used for procaspase3 primary antibody detection according to the manufacturers' protocol. DAPI and Hematoxiline were used to stain nuclei in IF and IHC, respectively. The samples were visualized with a fluorescent microscope (Olympus, Japan) at 400X magnification. The quantification of the immunohistochemical assay was based on the fluorescence intensity obtained by Image J software. DAB-precipitate thickness was measured with Image J software based on nuclear stain which indicated positive reaction to GFAP marker.

PDH and LDH activity

The enzyme activity of pyruvate dehydrogenase (PDH) and lactate dehydrogenase(LDH) were measured within 24 h following the ischemia with pyruvate dehydrogenase enzyme activity microplate assay kit (Abcam, Cambridge, MA) and lactate dehydrogenase assay kit (Colorimetric) ((Abcam, Cambridge, MA), respectively, which were implemented according to the protocol recommended by their manufacturers. Homogenized ischemic brain hemispheres were loaded on a plate and incubated in the assay solution. The optical density at 450 nm (OD 450) was measured for 20 min by Elisa reader (EX800, USA). The slope of the standard curve was calculated based on serial dilution and used to calculate the sample concentration.

Statistical analyses

All data were analyzed by GraphPad Prism v7.04. Analysis of variance (ANOVA) was used where appropriate for comparison among the different groups followed by a post hoc test (Tukey's test) for multiple comparisons. All data were expressed as mean ± SD, and $P \leq 0.05$ was considered as the threshold of statistical significance.

Results

Cerebral infarct volumes

The appearance of white areas was described as the infarcted brain tissue, and red areas were defined as the non-infarcted region in the right hemisphere of ischemic rats (Fig. 1a). The quantitative comparisons of total cerebral infarction volumes are shown in Fig. 1b. As shown in Fig. 1a-b, no significant difference was found between Veh and MCAO groups ($P > 0.05$) when stained with TTC. Within 24 h after reperfusion, the infarct volume of the brain tissue in the E group was 14.13% lower than in the Veh group ($P < 0.05$). The infarct volume of the brain tissue in the E + M100 group was 44.3% lower than in the E group ($P < 0.0001$). The infarct volume of the brain tissue in the E + M100 group was 43.2% lower than in the M10 group ($P < 0.0001$). The infarct volume of the brain tissue in the E + M100 group was 35.74% lower than in the E + M10 group ($P < 0.0001$).

Brain water content

To evaluate the anti-inflammatory effects of ethanol and modafinil, brain water content (Fig. 1c) was measured within 24 h after reperfusion in different groups. The brain water content of the E group was 2.1% lower than in the MCAO group ($P < 0.0001$).The brain water content in the M100 group was 1.92% lower than in the E group($P < 0.0001$). The brain water content in the M100 group was 1.92% lower than in the M10 group ($P < 0.0001$). Finally, the brain water content in the M100 group was 2.07% lower than in the E + M10 group ($P < 0.0001$).

Cresyl violet staining

Dark cells (dead cells) were counted by Cresyl violet staining (Fig.2a-i) within 72 h after reperfusion in different groups. Quantitative analysis (Fig.2j) shows that the MCAO group had the highest number of dark cells (92 ± 2) which was not significantly different from that of the Veh (89.6 ± 6.64) and E (90.6 ± 5.64) groups($P > 0.05$). However, the dark cells in the E + M100 (47.2 ± 7.4) group was significantly lower than the E, M10 (82.6 ± 5.4) and E + M10 (89.4 ± 5.8) groups ($P < 0.0001$).

Terminal dexynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL-positive cells (apoptotic cells) (Fig. 3a-i) were measured in different groups 72 h after the reperfusion. Quantitative analysis (Fig.3j) shows that pretreatment

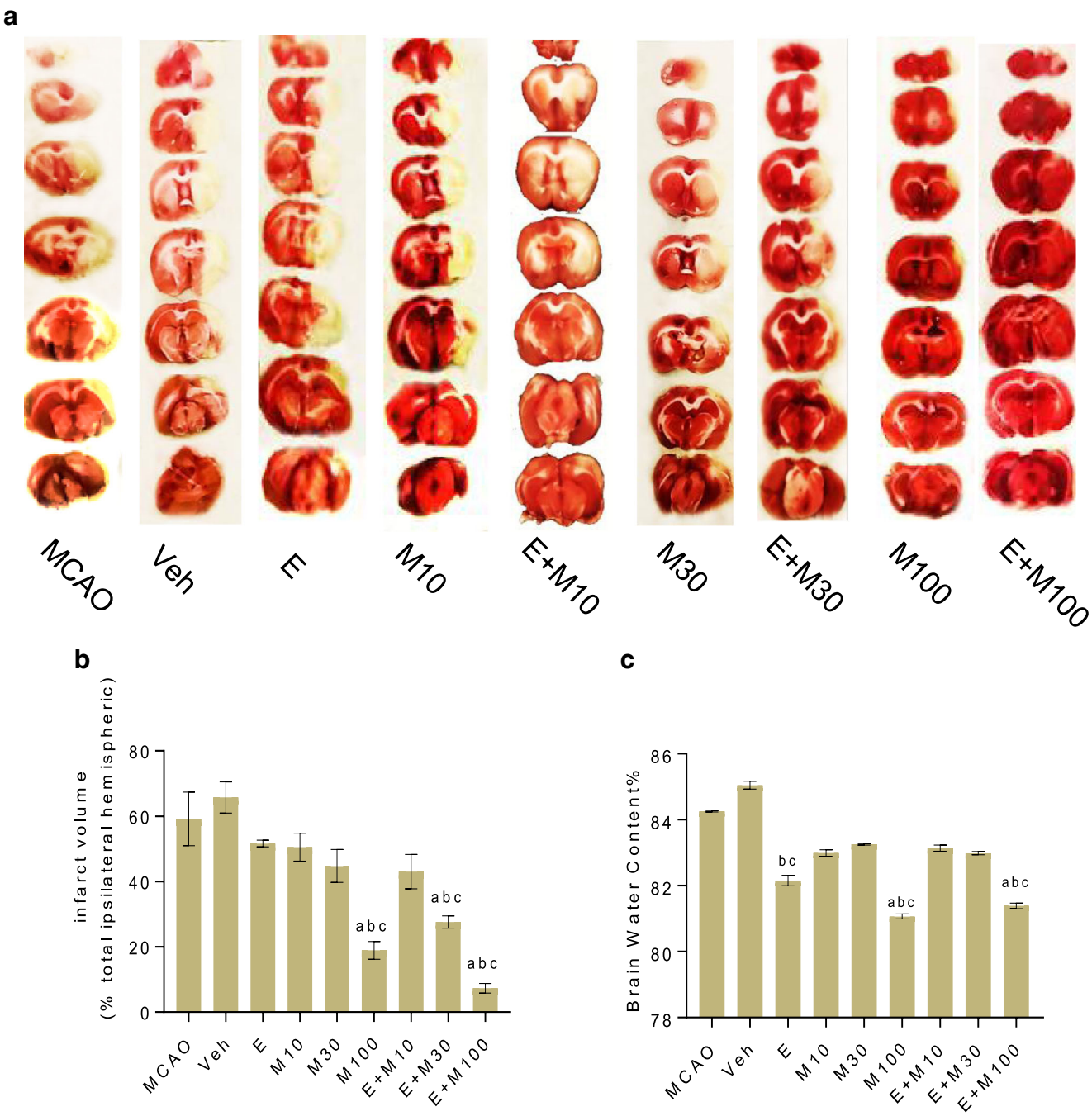

Fig. 1 Comparison of Cerebral infarct volume and edema 24 h after MCAO, **(a)** Cerebral tissues were coronally sectioned into 2-mm-thick slices and stained with triphenyltetrazolium chloride 24 h after occlusion of middle cerebral artery in different groups. Ischemic regions are colored white (light) and non-ischemic regions are red (dark). **(b)** Comparison of infarct volume in different groups. Data are given as arithmetic means ± SEMs **(C)** Comparison of brain edema 24 h after ischemia in different groups. Data are given as arithmetic means ± SEMs .$^{a}P \leq 0.05$ compared to E. $^{b}P \leq 0.05$ compared to E + M10. $^{c}P \leq 0.05$ compared to M10

with ethanol and modafinil reduced TUNEL-positive cells (apoptotic cells) 72 h after the reperfusion. The number of TUNEL-positive cells in the E group were 10% lower than the MCAO group ($P < 0.0001$). The number of these cells in the E + M100 group was 30.67% lower than the M10 group ($P < 0.0001$). Finally, it was 32.67% lower in the E + M100 group than in the E + M10 group ($P < 0.0001$).

Immunohistochemistry (GFAP marker)

72 h after reperfusion, GFAP-positive cells (Fig. 4a-i) were identified by immunocytochemistry and counted in different groups. Quantitative analysis (Fig. 4j) shows that the MCAO (71% ± 0.58) and Veh (75.66% ± 1.2) groups had the highest number of GFAP-positive cells.

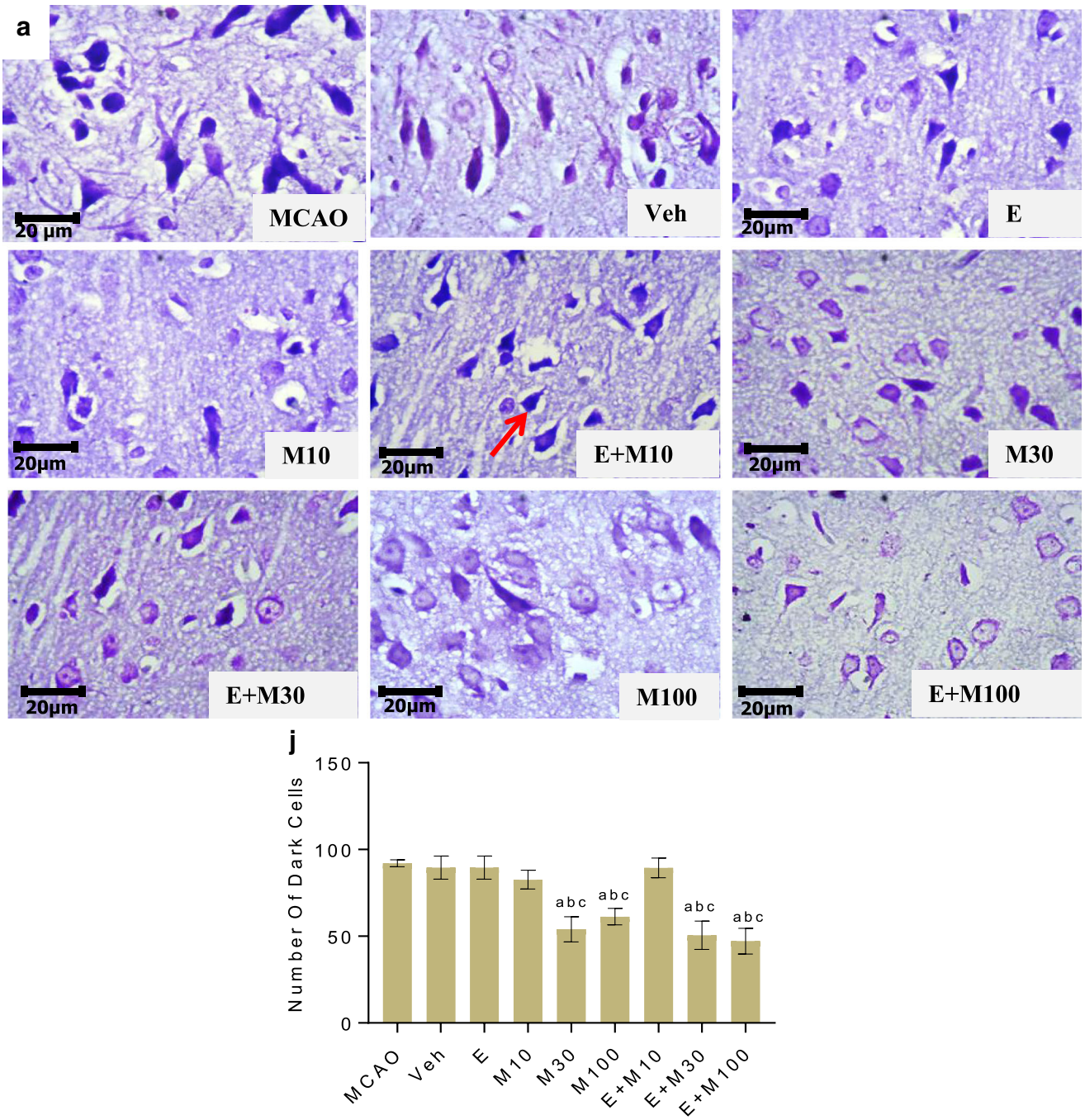

Fig. 2 Representative of penumbral cortex of ischemic hemisphere stained with cresyl violet 72 h after ischemia in different groups, **(a)** MCAO, **(b)** Veh, **(c)** E, **(d)** M10, **(e)**E + M10, **(f)** M30, **(g)** E + M30, **(h)** M100, **(i)**E + M100. Arrow indicates dead cell. Scale bars: 20 μm **(J)** Quantitative analysis of the number of dark cells in different groups. Data are given as arithmetic means ± SEMs . $^{a}P \leq 0.05$ compared to E. $^{b}P \leq 0.05$ compared to E + M10. $^{c}P \leq 0.\ 0.05$ compared to M10

The number of these cells in the E (60% ± 1.15) group was significantly lower than the MCAO and Veh groups ($P < 0.0001$). The number of GFAP positive cells in the E + M100 (16% ± 1.53) group was significantly lower than the E, M10 and E + M10 groups ($P < 0.0001$).

Immunohistochemistry (procaspase-3 marker)

48 h after reperfusion, procaspase-3-positive cells (Fig. 5a-i) were identified and counted by immunocytochemistry in different groups. Quantitative analysis (Fig.

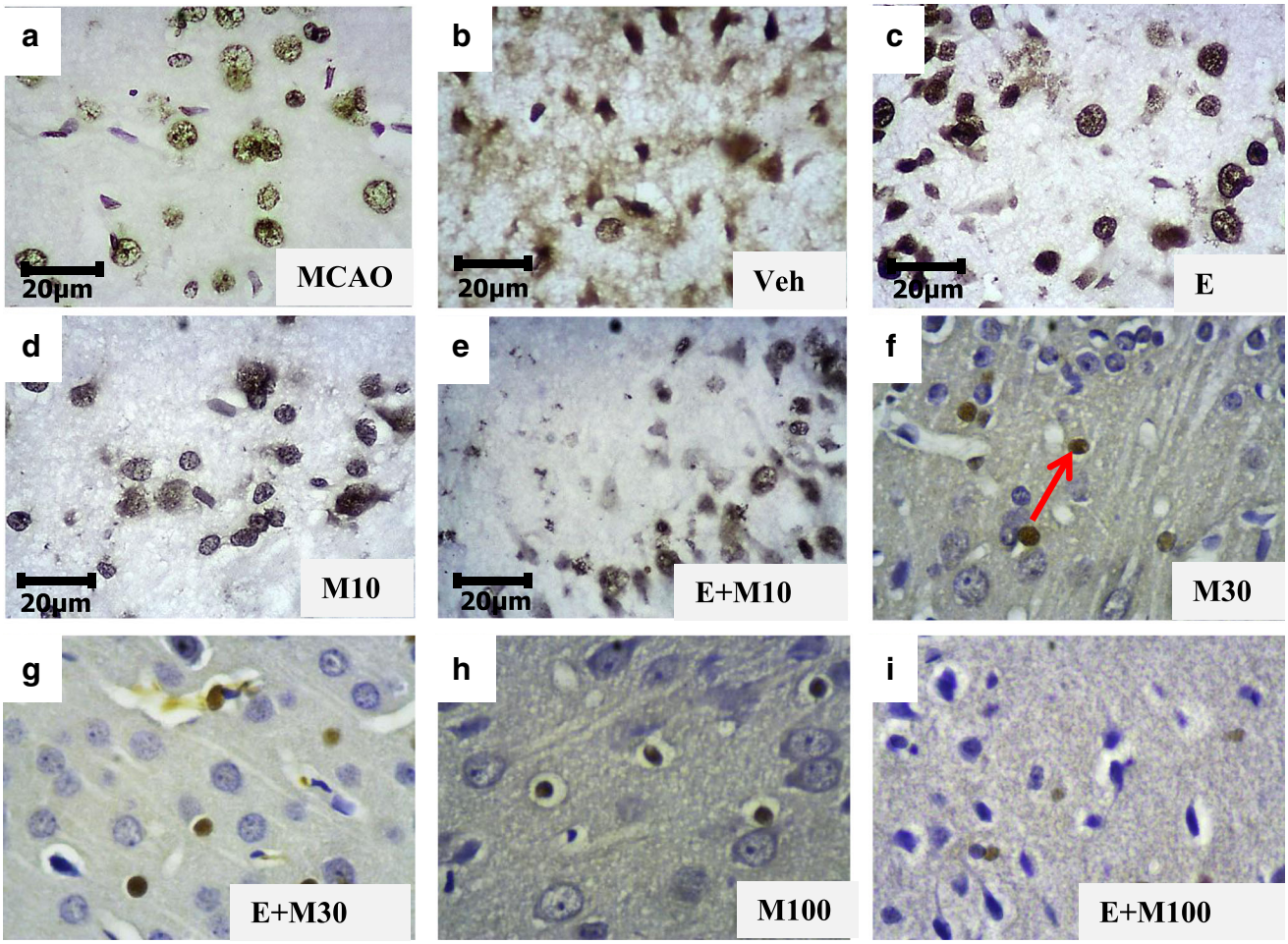

Fig. 3 Photomicrographs of TUNEL-positive cells in the penumbral cortex of ischemic hemisphere 72 h after ischemia in different groups, **(a)** MCAO, **(b)** Veh, **(c)** E, **(d)** M10, **(e)**E + M10, **(f)** M30, **(g)** E + M30, **(h)** M100, **(i)**E + M100. Arrow indicates dead cell. Scale bars: 20 μm **(j)** Quantitative analysis of cortical TUNEL-positive cells in different groups. Data are given as arithmetic means ± SEMs .$^{a}P \leq 0.05$ compared to E. $^{b}P \leq 0.05$ compared to E + M10. $^{c}P \leq 0.05$ compared to M10

5j) shows that the MCAO (79.66% ±0.33) and Veh groups (77.66% ± 0.88) had the highest number of procaspase-3-positive cells. The number of procaspase-3-positive cells in the E group (46.66% ± 1.45) were significantly lower than the MCAO and Veh groups ($P < 0.0001$). The number of these cells in the E + M100 (12.33% ± 1.45) group was significantly lower than the E, M10, E + M10 groups ($P < 0.0001$).

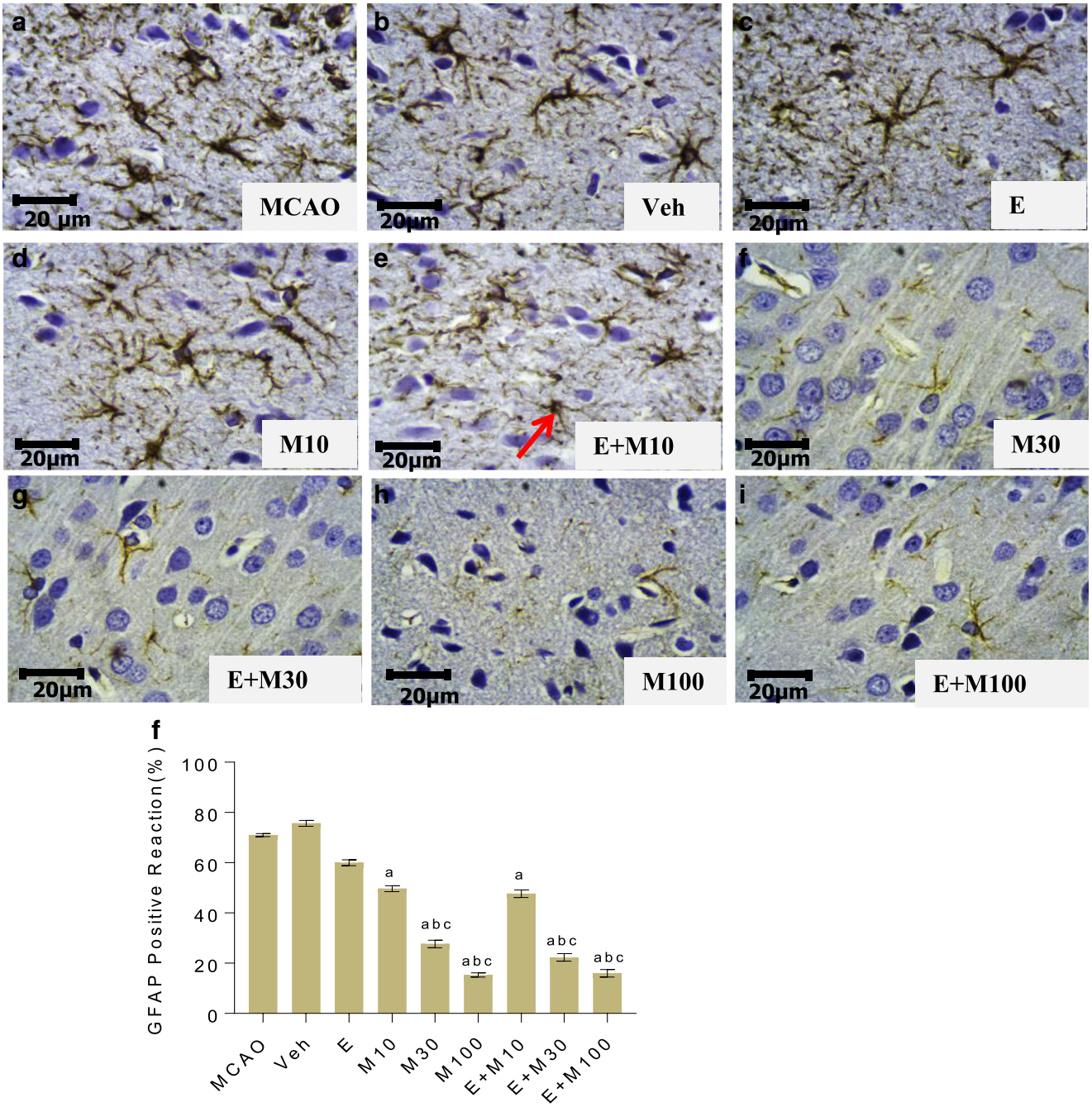

Fig. 4 Immunohistochemical staining of GFAP(astrocyte) in the penumbral cortex 72 h after ischemia in different groups, **(a)** MCAO, **(b)** Veh, **(c)** E, **(d)** M10, **(e)**E + M10, **(f)** M30, **(g)** E + M30, **(h)** M100, **(i)**E + M100. Arrow indicates GFAP positive cells. Scale bars: 20 μm **(J)** Quantitative analysis of GFAP-positive cells in different groups. Data are given as arithmetic means ± SEMs .$^{a}P \leq 0.05$ compared to E. $^{b}P \leq 0.05$ compared to E + M10. $^{c}P \leq 0.$ 0.05 compared to M10

PDH activity

24 h after reperfusion, PDH enzyme activity (Fig. 6a) was identified and measured. PDH enzyme activity in the E group (5.77 ± 0.69) was not significantly different from the MCAO (5 ± 0.66) and Veh(4.66 ± 1.45) groups ($P > 0.05$). PDH enzyme activity in the E + M100 (19 ± 2.72) group was significantly higher than the E, M10 (4 ± .66) and E + M10 (7.88 ± 1.26) groups ($P < 0.0001$).

LDH activity

24 h after reperfusion, LDH enzyme activity (Fig. 6b) was identified and measured. LDH enzyme activity in

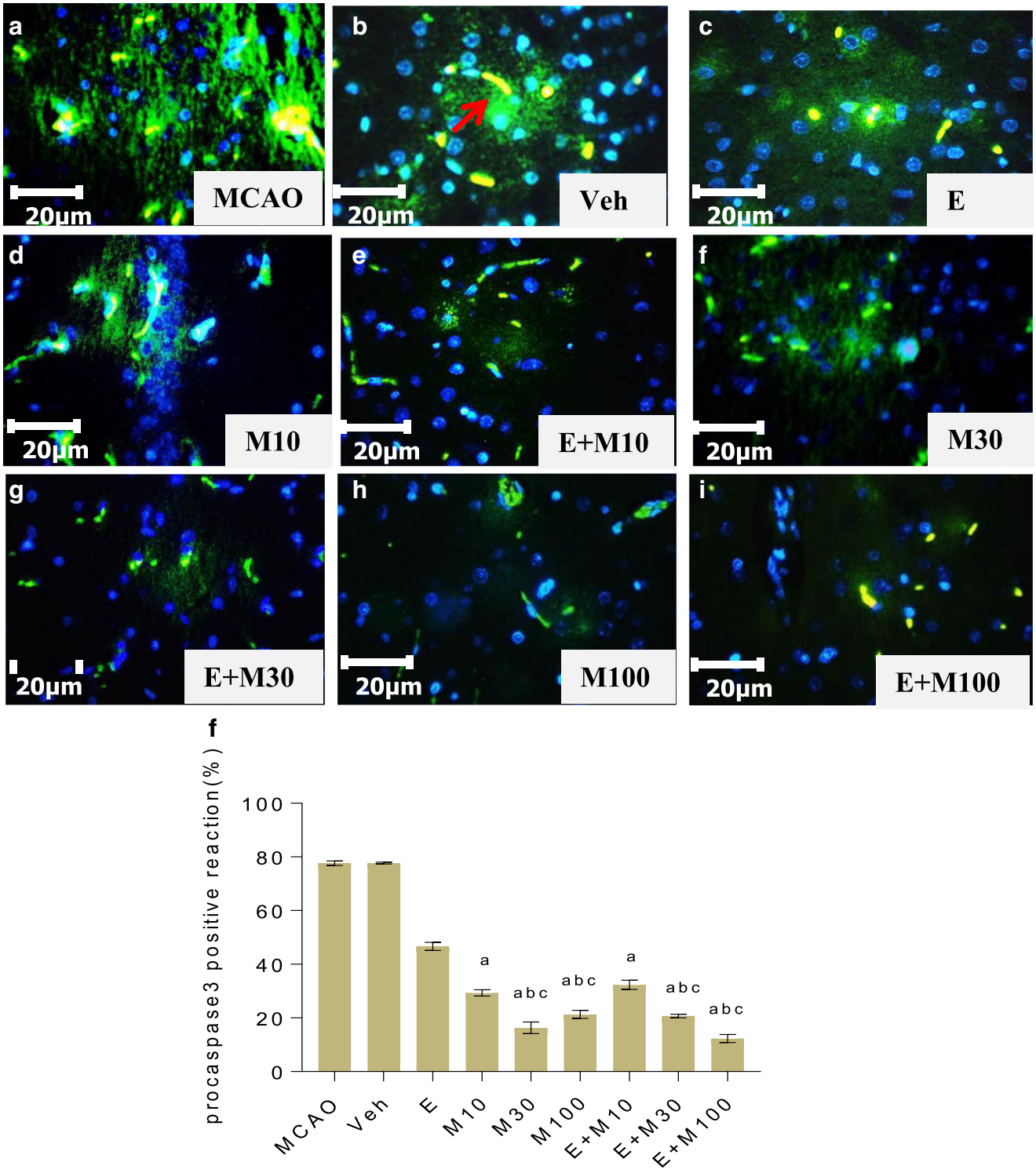

Fig. 5 Immunohistochemical staining of procaspase3 in the penumbral cortex 48 h after ischemia in different groups, **(a)** MCAO**, (b)** Veh, **(c)** E, **(d)** M10**, (e)**E + M10, **(f)** M30, **(g)** E + M30**, (h)** M100, **(i)**E + M100. Arrow indicates procaspase3 positive cells. Scale bars: 20 μm **(J)** Quantitative analysis of procaspase-3-positive cells in different groups. Data are given as arithmetic means ± SEMs . $^{a}P \leq 0.05$ compared to E. $^{b}P \leq 0.05$ compared to E + M10. $^{c}P \leq 0.$ 0.05 compared to M10

the E group (4.92 ± 0.24) was significantly lower than the MCAO (6.22 ± 0.09)($P < 0.0001$) and Veh (6.17 ± 0.06)($P = 0.0002$) groups. LDH enzyme activity in the E + M100 (0.16 ± 0.15) group was significantly lower than the E, M10 (2.55 ± 0.4), and E + M10 (2.55 ± 0.4) groups ($P < 0.0001$).

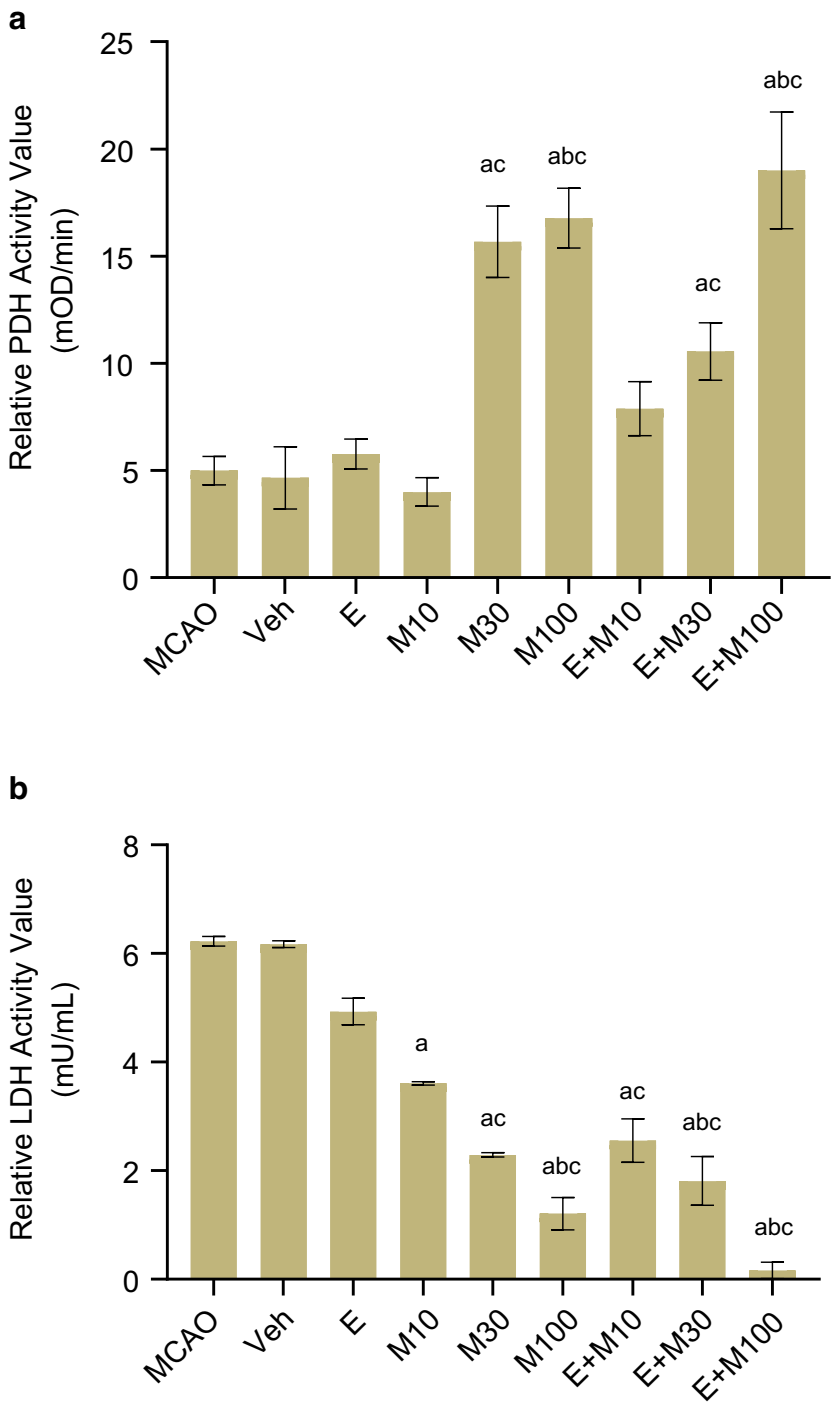

Fig. 6 Assessment of PDH and LDH activity 24 h after ischemia in different groups, **(a)** Quantitative analysis of PDH activity level(mOD/min) 24 h after ischemia in different groups. **(b)** Quantitative analysis of LDH activity(mOD/min) 24 h after ischemia in different groups. Data are given as arithmetic means ± SEMs. [a]$P \leq 0.05$ compared to E. [b]$P \leq 0.05$ compared to E + M10. [c]$P \leq 0.$ 0.05 compared to M10

Discussion

The present study investigated the neuroprotective effect of the single usage of ethanol and modafinil (in a dose-dependent manner) along with the combination of both compounds in transient focal cerebral ischemia in rats. It was shown that both brain damage and edema were decreased. Additionally, ethanol alone treatment, modafinil (in a dose-dependent manner), and their combination inhibited apoptosis of cortical neurons and reduced the glial scars (GFAP-positive marker) as well as pro-apoptotic factor (procaspase-3) following transient focal cerebral ischemia. Furthermore, while the activity of LDH was decreased, increased activity of PDH was observed in response to the above treatments.

Heavy and light-to-moderate alcohol consumption may have dual effects on the J-shaped pattern. Hence, light-to-moderate alcohol consumption decreases the mortality and infarct volume following cerebral ischemia(McCarter et al.

2017a). Several studies have reported that treatment with 1.5 g/kg of intraperitoneal injection of ethanol reduced the total infarct volume by 40%–47% as compared with the control group of ischemic rats (Kochanski et al. 2013; Wang et al. 2012). McCarter et al. reports that pretreatment with red wine and ethanol (1.4 g/kg/day) via gavage for eight weeks decreased the total infarct volume (19%) at 24 h after the induction of focal cerebral ischemia. Also, neuroprotective effects of low-dose ethanol are not dependent upon the type of alcohol administration(McCarter et al. 2017a). Stevenson et al. demonstrate that combination of ethanol (1 g/kg) and normobaric oxygen (NBO) injection through the right femoral artery immediately after t-PA infusion reduced infarct volume by about 10.4% (Cai et al. 2016a). In the present study, treatment with 1.5 g/kg of intraperitoneal ethanol immediately reduced the total infarct volume by 7.5% after the ischemia. However, the decrease in the infarct volume was not significant. This finding was unexpected and suggests that a single injection of ethanol may not significantly reduce the total infarct volume.

The protective effect of modafinil has been studied in animal models of neurodegenerative diseases, but the underlying mechanism is not fully understood (Bibani et al. 2012). Ueki et al. reports that modafinil treatment in a dose-dependent manner (10, 30, and 100 mg/kg i.p.) 7 days before the ischemia reduced the lesion volume (Ueki et al. 1993). In our study, we showed that treatment with modafinil at doses of 10, 30, and 100 mg/kg reduced the total infarct volume by 8.6%, 14.4%, 40.2%, respectively, after the ischemia. The obtained results were consistent with the findings in the literature. According to our study, the combination of modafinil at doses of 10, 30, and 100 mg/kg and ethanol at a dose of 1.5 g/kg reduced the total infarct volume by 16.1%, 31.6%, 51.8%, respectively. The results suggest that the combination of ethanol and modafinil strengthens the synergistic neuroprotective effect of these compounds on cerebral ischemia.

The formation of edema plays a significant role in neuronal death and development of brain lesion after stroke. Edema is able to spread the lesions through increasing intracranial pressure and reducing the blood flow to the brain tissue(Lundgren et al. 1991; Murakami et al. 1997). Previous studies report that a low dose of ethanol (2 g/kg) could decrease, and a higher dose (3 g/kg) could increase, brain edema. However, the lower dose of ethanol (0.2–0.65 g/kg, circulating in blood vessels at a dose of 46 mg/dL) demonstrated no neuroprotective potential after the stroke (Aronowski et al. 2003; Strong et al. 2000). Other studies demonstrated that both red wine and ethanol significantly reduced the blood pressure (McCarter et al. 2017a). Ueki et al. report that modafinil, as applied in a dose-dependent manner, prevented ET-l-induced increase in perfusate lactate levels without affecting the ET-1-induced reduction of striatal blood flow (Ueki et al. 1993). Modafinil at doses of 300 mg/kg and 600 mg/kg does not increase the cortical cerebral blood flow in rats (Florence et al. 2000). These studies proposed that ethanol in particular doses can reduce blood pressure and prevent brain edema, leading to better functional recovery in patients with acute ischemic stroke (Chamorro et al. 1998). The reduction in blood pressure by ethanol is probably associated with the suppressed inflammatory responses after the ischemia (McCarter et al. 2017a) while modafinil, in contrast to ethanol, does not affect the reduction of blood flow (Ueki et al. 1993). In the current study, a significant decrease in water content was observed in the E group compared to the MCAO group, thereby providing clear evidence of the efficacy of ethanol against the formation of brain edema following an ischemic stroke. However, a large decrease in water content was observed in the M100 and E + M100 groups when compared to the E group, showing that modafinil at a dose of 100 mg/kg, as well as the combination of modafinil (100 mg/kg) + ethanol(1.5 g/kg), is able to decrease the edema. The effect of ethanol in decreasing edema was lower than modafinil (100 mg/kg).

Development of cerebral ischemia leads to increased production of free radicals and decreased activity of antioxidant enzymes. These processes activate apoptosis pathway which, in turn, increases cellular damage and apoptosis(Buga et al. 2008; Doyle et al. 2008; Women's Hospitals 2009). Kanbak et al. demonstrated that moderate alcohol consumption has protective effects via the inhibition of lysosomal protease release and nitric oxide production on apoptotic cell death after traumatic brain injury (Kanbak et al. 2013). Hafeez et al. demonstrated that ethanol (1.5 g/kg) is able to decrease the expression of pro-apoptotic proteins such as PKC-δ when administered within 3 to 24 h after reperfusion. Contrarily, it can increase the anti-apoptotic factors, such as Akt, at both levels of mRNA and protein following an ischemic stroke(Hafeez et al. 2014). Su et al. indicated that the neuroprotective effect of preconditioning with low doses of ethanol is mediated by counteracting the elevation of cytosolic Ca 2 + (Su et al. 2017). Recent studies have indicated that treatment with modafinil prevents inflammation and loss of dopaminergic neurons in substantia nigra in animal models of Parkinson's disease which were induced by administration of a neurotoxin called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)(Aguirre et al. 1999; Fuxe et al. 1992; Xiao et al. 2004). Modafinil may target the mitochondrion, as a source of generating reactive oxygen species in the cell, to directly inhibit the formation of free radicals. Modafinil may enhance the ability of cytochrome c (in the mitochondrial electron transport chain) to accept and donate electrons that would directly reduce the net hydrogen peroxide levels, superoxide production, and MPTP-induced neuronal death (Gerrard and Malcolm 2007). Modafinil prevents methamphetamine-induced toxicity via increasing pro-apoptotic proteins such as BAX and decreasing anti-apoptotic factors such as Bcl-2 to provide a protective effect against dopamine toxicity, cell

death, and, neuroinflammation in the striatum of rats (Raineri et al. 2012).

In the present study, ethanol (1.5 g/kg) decreased TUNEL-positive cells. Moreover, modafinil (in a dose-dependent manner) caused a significant decrease in TUNEL-positive cells in comparison to ethanol. Hence, it would be plausible that the combination of ethanol and modafinil could enhance the synergistic neuroprotective effect of both compounds which results in decreased neuronal death. Cresyl violet staining demonstrated that ethanol could not decrease dark cells (apoptotic cell) when administered alone. Worthy of note is that modafinil at doses of 30 and 100 mg/kg as well as its combination with ethanol is capable of providing protection against cell death in comparison with ethanol-alone treatment (Fig. 3).

After tissue processing and the addition of primary antibody, FITC anti-rabbit-conjugated secondary antibodies (green-fluorescent dye for the detection of procaspase3 positive cells) and DAPI (blue-fluorescent dye extensively used to detect nuclei) were applied for counterstaining(Svandova et al. 2018). Positive procaspase-3 cells were observed as green cells that possessed blue nucleus. Caspase-3 is considered a key molecule which exists in the cell as a low-activity zymogen. Procaspase-3 is activated by proteolysis to form caspase-3 (Wang et al. 2014). In the present study, ethanol(1.5 g/kg) decreased the number of procaspase-3-positive cells. Modafinil (applied in a dose-dependent manner) caused a significant decrease in procaspase-3-positive cells as compared with ethanol. Notably, the combination of ethanol and modafinil enhances the synergistic neuroprotective effect by reducing the number of procaspase-3-positive cells (Fig. 5).

After tissue processing and the addition of primary antibody, Peroxidase-conjugated secondary antibodies with chromogen/substrate diaminobenzidine were used to visualize GFAP positive cells(Davis et al. 2002). The positive cells were visualized as brown dye possessing end feet. In the ischemic region, GFAP-positive cells begin to increase within 30 min. They reach the maximum value within 1–3 days. After seven days, the number of cells will begin to decrease, and after 28 days they are likely to reach the baseline (Jin et al. 2010). The gap junctions of astrocytes may remain open after the ischemia, allowing proapoptotic factors to spread through the syncytium and expand the size of the infarct (Lin et al. 1998; Martinez and Saez 2000). There is a large body of evidence that decrease in astrogliosis is congruous with reduction in infarct size (Wang et al. 2008). Soon after MCAO, the injured neurons in the core and penumbra produce proinflammatory factors, cytokines, and reactive oxygen species, which stimulate the activation of both astrocytes and microglia (Tuttolomondo et al. 2008). Penumbra is a small area adjacent to the core of the ischemic region that progresses to infarction over the time (Carmichael 2005). Activated astrocytes can produce proinflammatory cytokines and interferons that can directly induce apoptosis of neuronal cells following the ischemia(Barreto et al. 2011). It has been demonstrated that the treatment with modafinil (in a dose-dependent manner) decreases the volume of neostriatum ischemia lesion through a reduction in glial fibrillary acidic protein (Ueki et al. 1993). Another study reports that modafinil counteracts with methamphetamine-induced astrocyte activation (Raineri et al. 2012). Sleep deprivation promotes the processes of hypertrophied astrocytes characterized by enhanced GFAP immune-reactivity that is attenuated by modafinil treatment (Sahu et al. 2013).

In the present study, ethanol (1.5 g/kg) decreased GFAP-positive cells. Furthermore, modafinil (in a dose-dependent manner) decreased GFAP-positive cells more significantly in comparison with ethanol. According to previous studies, both ethanol and modafinil are able to decrease the activated astrocytes (Raineri et al. 2012; Sahu et al. 2013; Ueki et al. 1993) and, eventually, decrease the proinflammatory cytokines (Barreto et al. 2011) and chemokines(Kim 1996; Sofroniew 2000) produced from astrocytes. Such phenomena decrease the spread of proapoptotic factors (Lin et al. 1998; Martinez and Saez 2000). It is, however, thought that modafinil is a more powerful neuroprotective agent than ethanol. As a consequence, combination of ethanol and modafinil is highly likely to enhance the synergistic neuroprotective effect and decreases GFAP-positive cells (Fig. 4).

Investigating the modulation of PDH mechanism in the process of ischemia would open up a new perspective on understanding the molecular events involved in neuroprotection after the ischemia (Cai et al. 2016b). Pyruvate dehydrogenase complex (PDH) is located in the mitochondrial matrix and catalyzes the conversion of pyruvate to acetyl coenzyme, making a bridge between anaerobic and aerobic energy metabolism. The overproduced ROS inactivates PDH enzyme during ischemia-induced oxidative stress (Richards et al. 2006), leading to limited oxidative phosphorylation and contributing to further ROS production and damage to proteins, lipids, and nucleic acids(Pradeep et al. 2012).

The oxygen and glucose levels increase during reperfusion, causing overproduction of reactive oxygen species (ROS) and further damage (Kochanski et al. 2013). LDH converts pyruvate to lactate during the hypoxic conditions. The accumulation of lactate occurs in acidosis and cell death (Rossignol et al. 2003; Siesjo 1988).

A low dose of ethanol consumption has a depressive effect on cerebral metabolism and reduction of both glucose utilization and ATP consumption in healthy individuals (Volkow et al. 1990; Volkow et al. 2006). Ethanol may act as a neuroprotective agent to limit the oxidative stress through decreasing mitochondrial generation of ROS that inhibits brain glucose metabolism (Kochanski et al. 2013; Volkow et al. 1990). This agent may have neuroprotective effects against NMDA

receptor (a subtype of glutamate receptor) excitotoxicity after the stroke (Chandler et al. 1993). The neuroprotective effect of ethanol may be mediated by PDH enzyme to improve oxidative phosphorylation that restores ATP supplies and promotes metabolic recovery and cell survival. The combination of ethanol and normobaric oxygen (NBO) increases PDH activity as well as the protein expression of the enzyme and reduces ROS generation (Cai et al. 2016b). High-to-moderate doses of ethanol have also been shown to attenuate hyper-glycolysis and confer neuroprotection in traumatic brain injury (Kelly et al. 2000). Ueki et al. reported that modafinil treatment counteracted with the increase in perfusate lactate levels in ET-1-induced striatal ischemia. However, pyruvate outflow was not modulated (Ueki et al. 1993).

In this study, the activity of PDH and LDH in the E-alone group was not significantly different from that of the MCAO group and was not as effective as modafinil in terms of neuroprotective properties, suggesting that chronic treatment with ethanol might be effective (McCarter et al. 2017a; McCarter et al. 2017b) On the other hand, in the E + M100 group, the highest level of activity was observed for pyruvate dehydrogenase and the lowest level was observed for LDH, suggesting that combination of modafinil and ethanol enhances the neuroprotective effect.

Conclusion

In conclusion, our findings confirm that pretreatment with a combination of M100 and E is a beneficial strategy for protection of the brain against ischemic injury. It should be noted that combination of ethanol and modafinil indicates a similar mechanism of action that involves PDH and LDH to mediate the restoration of aerobic metabolism and to inhibit the oxidative injury in the reperfusion process.

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Compliance with ethical standards

Conflicts of interests Conflict of interest the authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of ethics Committee of Iran University of Medical Sciences (ethical code: ir.iums.rec 1395.9221313204).

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